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N-Acetyl Cysteine Blocks Mesangial VCAM-1 and NF-κB Expression *in Vivo*

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Inducible vascular cell adhesion molecule-1 (VCAM-1) in glomerular mesangial cells (GMC) exposed to lipopolysaccharide (LPS) *in vitro* involves the activation of nuclear factor-κB (NF-κB) and its interaction with the proximal VCAM-1 promoter. We used a murine model to assess the effect of the antioxidant, *N*-acetyl cysteine on GMC activation *in vivo*. Single intraperitoneal administration of *N*-acetyl cysteine completely suppressed LPS-induced VCAM-1 expression on the GMC surface. When an oligonucleotide spanning the NF-κB binding region of the VCAM-1 promoter was incubated with extracts from the renal cortex of LPS-treated animals, a single nucleoprotein complex formed. This complex was composed of p50 and p65, but not p52, c-Rel, or RelB, and its formation was dramatically inhibited by pretreatment with *N*-acetyl cysteine. D,L-Buthionine-[S,R]-sulfoximide, a compound that depletes glutathione, augmented VCAM-1 expression inducible with a suboptimal amount of LPS to levels comparable with using 50 μg of LPS alone. D,L-Buthionine-[S,R]-sulfoximide also potentiated the p50-p65 binding activity induced with a suboptimal amount of LPS. These data provide a redox-sensitive, transcriptional link between NF-κB and VCAM-1 in GMC *in vivo* and implicate oxidative stress as an important regulatory signal in the pathogenesis of glomerular mesangial cell disorders. *(Am J Pathol 1997, 151:1225–1229)*

Vascular cell adhesion molecule-1 (VCAM-1), a member of the immunoglobulin gene superfamily of adhesion molecules, is induced at the glomerular mesangial cells (GMC) surface after macrophage activation and immune complex deposition in murine models of autoimmune glomerular nephritis.[1] VCAM-1 is also expressed in GMC of the renal cortex of mice challenged intravenously with endotoxin[2] and in cultured mouse or rat GMC exposed to the cytokines, interleukin-1β and tumor necrosis factor-α.[3] Tumor necrosis factor-α activation of VCAM-1 transcription in cultured GMC[3] and vascular endothelial cells[4] requires the induction of nuclear factor-κB (NF-κB) and its interaction with the VCAM-1 promoter. NF-κB is a ubiquitously expressed multisubunit transcription factor family activated in multiple cell types by a large and diverse group of growth-regulatory molecules.

NF-κB activation can be specifically inhibited *in vitro* by antioxidants such as *N*-acetyl cysteine (NAC)[5] and pyrrolidine dithiocarbamate.[6] These antioxidants attenuate oxygen radical formation thus maintaining NF-κB in the inactive cytosolic form. NAC has been well-characterized *in vitro* and *in vivo* as an agent that protects against oxygen radical damage by direct scavenger properties and its ability to modulate intracellular glutathione levels.[7] This has led to the hypothesis that oxygen radicals regulate NF-κB activation through reduction-oxidation (redox) mechanisms.[8] Although Shan et al[9] have found that redox mechanisms influence collagen type I and IV transcription in cultured GMC via changes in intracellular redox potential, the involvement of redox mechanisms in renal pathophysiological processes *in vivo* is not entirely clear. To determine whether modulation of NF-κB activity *in vivo* is a bidirectional redox process, we used NAC and the glutathione-depleting agent, D,L-Buthionine-[S,R]-sulfoximide (BSO),[10] in a murine model of endotoxemia.

Materials and Methods

Administration of Lipopolysaccharide, NAC, and BSO

Pathogen-free mice of both sexes (CD1 and BALB/c) were purchased from the Charles River Breeding Laboratories (Wilmington, MA) and used after reaching a body mass of 22 to 24 g (approximately 8 weeks old). All

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animals were handled and maintained in accordance with the Guidelines of the Committee on Care and Use of Laboratory Animal Resources (National Research Council). Groups of three to five animals were injected intraperitoneally with 50 μg of *Escherichia coli* lipopolysaccharide (LPS, serotype 0.55:B5, Sigma) in phosphate-buffered saline (PBS) alone or 1 hour after a single intraperitoneal injection of NAC (Sigma) (1, 2, 4, 8, and 16 mg in PBS), BSO (Sigma) (8, 15, 23, and 30 μg in PBS), or PBS alone. Animals were killed 1 hour (tissue extracts) or 6 hours (immunoperoxidase staining) subsequently. No significant leukocyte adherence or infiltration is observed in this murine model of endotoxemia.[2]

Morphological and Immunoperoxidase Studies

VCAM-1 was immunolocalized using a monoclonal antibody, M/K-2, generously provided by Dr. P. W. Kincade (Oklahoma City, OK). An irrelevant, isotype-matched rat immunoglobulin (Sigma) was used as negative control at the same concentration. Kidneys were cut coronally, whereas the entire thoracic and abdominal aorta was dissected and cross-sectioned. Tissue sections were embedded in OCT, snap-frozen in liquid nitrogen, and 6-μm-thick frozen sections were cut and mounted on Vectabond-coated (Vector Laboratories, Burlingame, CA) glass slides. After air-drying for 30 minutes, sections were fixed in 100% ice-cold acetone for 7 minutes, air-dried for an additional 10 minutes, and stored at −70°C in plastic wrap until later use. VCAM-1 staining was carried out with the protocol previously described[2] using primary antibody at a 1/40 dilution for 1 hour at 22°C and a goat anti-rat IgG (heavy and light chain) peroxidase-conjugated antibody (mouse absorbed; Caltag, South San Francisco, CA) for 45 minutes in a humidified chamber. Binding was detected by the addition of 0.1 mol/L acetate buffer containing 3-amino-9-ethylcarbazole (Aldrich, Milwaukee, MI) in *N,N*-dimethylformamide and hydrogen peroxide.[2] Slides were counterstained in methyl green for 2 minutes and counterslipped with gelatin/glycerol. For histological evaluation, sections were fixed in methanol for 10 seconds and stained with hematoxylin (30 seconds) and eosin (10 seconds) before dehydration and coverslipping with Permount. Staining was scored + to ++++ (weak to strongly intense).

Preparation of Renal Tissue Extracts

Kidneys were placed on ice, the fibrous capsule surgically removed, and the cortex and medulla minced in ice-cold homogenization buffer A.[11] The suspension was frozen in liquid nitrogen, thawed at 22°C, and this freeze-thaw cycle repeated 4 times. The material was spun at 2000 rpm for 15 minutes at 4°C. The supernatant was combined with an equal volume of buffer C[18] and frozen at −70°C until use.

Electrophoretic Mobility Shift Assay

An oligonucleotide bearing both NF-κB binding sites in the VCAM-1 promoter[11,12] was ^{32}P-labeled with T_4 polynucleotide kinase (New England Biolabs) and used in electrophoretic mobility-shift assay (EMSA) as described previously.[3] To assure equal loading of extract, the concentration of protein in each sample was quantitated using the BioRad protein assay. For supershift analyses, polyclonal antibodies directed toward murine-specific NF-κB subunits were added 10 minutes before the addition of the probe.

Results

LPS-Induced VCAM-1 Expression on GMC is Inhibited by Pretreatment with NAC

Single administration of LPS (50 μg intraperitoneally) induced intense VCAM-1 staining (++++) on GMC as well as endothelial cells of the renal vessels and the aorta within 6 hours (Figure 1). VCAM-1 staining was also observed in the interstitium. Prior intraperitoneal injection of NAC (2 mg or greater) completely abolished VCAM-1 staining on the mesangial surface (Figure 1), whereas only partial inhibition (+/++) resulted when lower doses were used. NAC alone did not induce VCAM-1 expression (Figure 1). These doses of NAC, injected daily for up to 4 days, had no apparent side effects or appreciable histological abnormalities. Intraperitoneal doses of 16 mg or greater did result in mortality.

Activation of NF-κB and its Interaction with the Proximal VCAM-1 Promoter in Mice Injected with LPS and Effect of NAC

To investigate whether NF-κB is involved in the induction of VCAM-1 after LPS injection, we performed an EMSA with a ^{32}P-labeled oligonucleotide bearing the two NF-κB binding sites in the VCAM-1 promoter and extracts prepared from the renal cortex. A single nucleoprotein complex was detected 1 hour after the injection of LPS (Figure 2). To determine whether this complex contained NF-κB and the nature of the subunits involved, antipeptide polyclonal antibodies directed toward c-Rel, p50, IκB-α, p52, and p65 were used in the EMSA. The one complex was eliminated by p50 and p65 antibodies and unaffected by any of the other antibodies (Figure 2), indicating that the p50-p65 heterodimeric form of NF-κB is activated in the renal cortex of mice treated with LPS. Because VCAM-1 expression in GMC is dependent on the interaction of NF-κB with the proximal VCAM-1 promoter,[3] we hypothesized that NAC inhibits VCAM-1 expression by suppressing the activation of NF-κB. EMSA revealed that the antioxidant compromised the induction of NF-κB by LPS (Figure 2).

VCAM-1 Immunostaining on GMC by Low-Dose LPS is Enhanced by BSO

Injection of mice with 2.5 μg of LPS elicited a weak increase (+) in VCAM-1 expression (Figure 3). When this

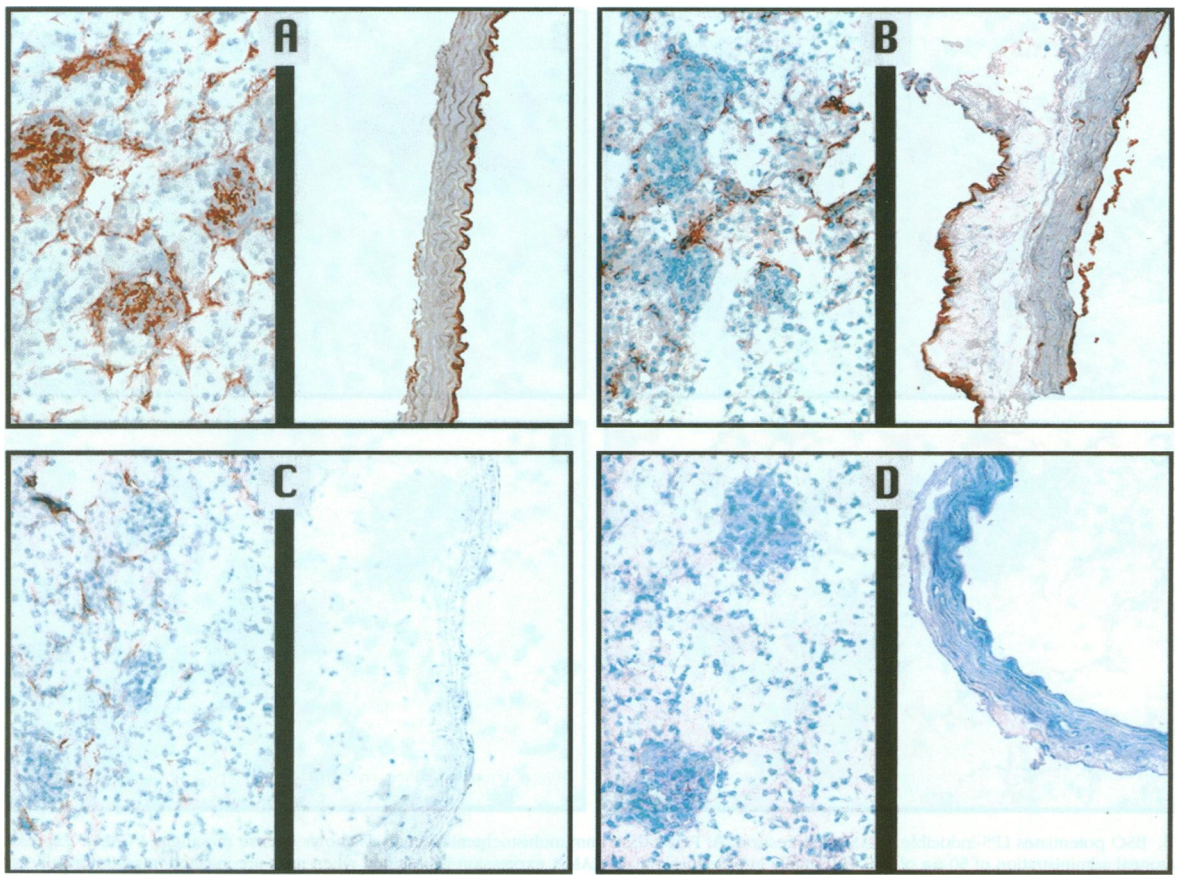

Figure 1. LPS-induced VCAM-1 expression *in vivo* is inhibited by NAC. **A:** Peroxidase immunohistochemical analysis showing mesangial VCAM-1 expression 6 hours after intraperitoneal administration of 50 μg of LPS (**left panel**). VCAM-1 staining is also detected on the surface of aortic endothelial cells (**right panel**). **B:** NAC pretreatment prevents LPS-induced mesangial VCAM-1 expression (**left panel**) but has no effect on endothelial VCAM-1 expression (**right panel**). **C:** NAC alone does not affect VCAM-1 expression on mesangial cells (**left panel**) or aortic endothelial cells (**right panel**). **D:** Injection of PBS alone does not induce VCAM-1 in mesangial cells (**left panel**) or endothelial cells (**right panel**). Histological plates are representative of VCAM-1 changes throughout each tissue examined and a consistent feature among the animals tested in each group.

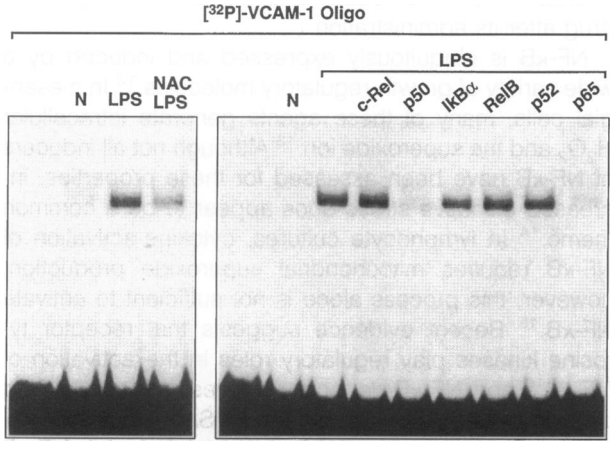

Figure 2. NF-κB is activated *in vivo* by LPS and binds to the proximal VCAM-1 promoter. EMSA using extracts prepared from renal cortex of mice injected intraperitoneal with 50 μg of LPS and a ^{32}P-labeled VCAM-1-oligonucleotide bearing the two NF-κB binding sites in the proximal VCAM-1 promoter. The formation of this complex is inhibited by pretreatment with NAC (**left panel**). The nucleoprotein complex is eliminated only when antibodies to p50 and p65 are used (**right panel**). The **first lane** represents radiolabeled oligonucleotide run without nuclear extract, as evidenced by the absence of a specific nucleoprotein complex with electrophoretic mobility slower than that of the probe itself.

low dose of LPS was preceded by a single intraperitoneal injection of BSO (8 or 16 μg), no significant difference in the staining intensity of VCAM-1 was observed (data not shown). However, intraperitoneal administration of 23 μg of BSO or greater dramatically increased low-dose LPS-induced VCAM-1 expression to levels comparable with 50 μg of LPS alone (++++) (Figure 3). VCAM-1 was also superinduced in vascular endothelial cells in mice pretreated with BSO.

BSO Augments LPS-inducible NF-κB Interaction with the VCAM-1 Promoter

EMSA, using extracts from renal cortices of mice injected with 2.5 μg of LPS and the ^{32}P-labeled VCAM-1 promoter fragment, revealed a weak induction of NF-κB (Figure 4). Administration of BSO alone failed to activate the transcription factor. However, treatment with 23 μg of BSO before injection of 2.5 μg of LPS resulted in a profound induction of NF-κB binding activity. This activation was comparable with that obtained using 50 μg of LPS alone (Figure 4). These findings taken together indicate that BSO potentiates, whereas NAC inhibits, the induction of NF-κB, its interaction with the proximal VCAM-1 pro-

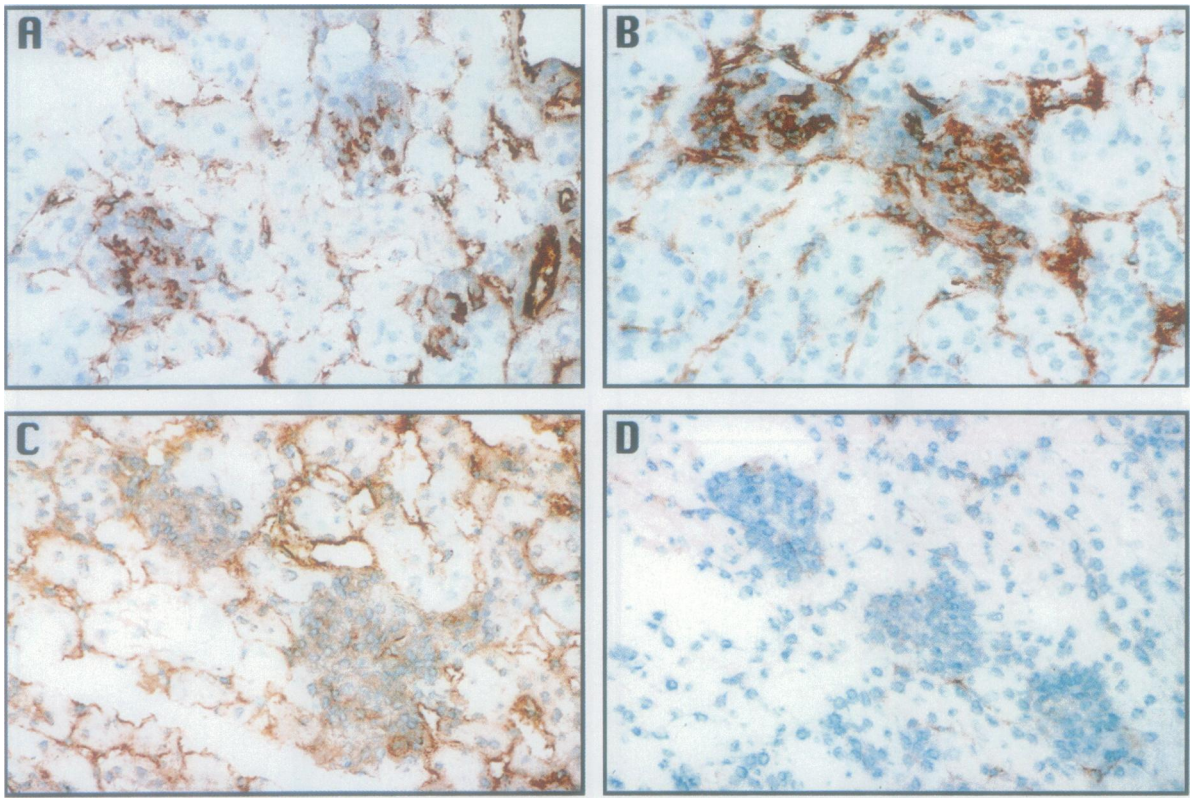

Figure 3. BSO potentiates LPS-inducible VCAM-1 expression. **A:** Peroxidase immunohistochemical analysis shows intense mesangial VCAM-1 expression after intraperitoneal administration of 50 μg of LPS. **B:** Equally intense mesangial VCAM-1 expression is obtained when mice are injected intraperitoneally with 8 mg of BSO followed 1 hour later by 2.5 μg of LPS. **C:** Weak mesangial VCAM-1 staining after administration of 2.5 μg of LPS alone. **D:** BSO alone does not induce mesangial VCAM-1 expression. Histological plates are representative of VCAM-1 changes throughout each tissue examined, and a consistent feature among the animals tested in each group.

moter, and the expression of VCAM-1 on the glomerular surface.

Discussion

NF-κB, on activation, accumulates in the nuclei of cultured murine and rat mesangial cells in which it binds to specific sites in the proximal VCAM-1 promoter and drives expression of the VCAM-1 gene.[3] The present study used a murine model of endotoxemia to demonstrate that LPS-inducible VCAM-1 expression at the glomerular mesangial surface is preceded by the interaction of NF-κB p50-p65 heterodimers with the VCAM-1 promoter. Pretreatment of the mice with NAC, a radical scavenger and inducer of glutathione production, abolished both the interaction of NF-κB with the VCAM-1 promoter and VCAM-1 expression on the GMC surface. Conversely BSO, a specific inhibitor of γ-glutamylcysteine synthetase, catalyst of the rate limiting step in glutathione synthesis, enhanced both NF-κB and VCAM-1 activation after injection of a suboptimal dose of LPS. Interestingly, NAC also inhibited endotoxin-induced VCAM-1 expression in the interstitial microvasculature. This is consistent with the requirement of NF-κB in LPS-inducible endothelial VCAM-1 expression.[5] Our inability to observe the inhibitory effect of NAC on LPS-induced VCAM-1 expression in aortic endothelium was probably because of differences in the distribution and localized activity of the drug after its administration.

NF-κB is ubiquitously expressed and induced by a wide variety of growth-regulatory molecules.[12] In mesangial cells, many of these agents generate intracellular H_2O_2 and the superoxide ion.[13] Although not all inducers of NF-κB have been assessed for these properties, increased oxidative stress does appear to be a common theme.[14] In lymphocyte cultures, cytokine-activation of NF-κB requires mitochondrial superoxide production, however, this process alone is not sufficient to activate NF-κB.[15] Recent evidence suggests that receptor tyrosine kinases play regulatory roles in the activation of NF-κB[16] and NF-κB-dependent genes[17] by modulating oxidant levels.[18] Our results from EMSA and immunohistochemistry indicate that NAC can inhibit inducible NF-κB and VCAM-1 expression, whereas the induction of both these molecules was superinduced by BSO. These opposite effects provide strong evidence that reactive oxygen intermediates mediate NF-κB and VCAM-1 activation in mesangial cells.

NAC has been used as a therapeutic agent in a variety of clinical settings. For example, NAC serves as a mucolytic agent in chronic bronchitis,[19] an antidote in acetaminophen-induced hepatotoxicity,[20] and as an inhibitor

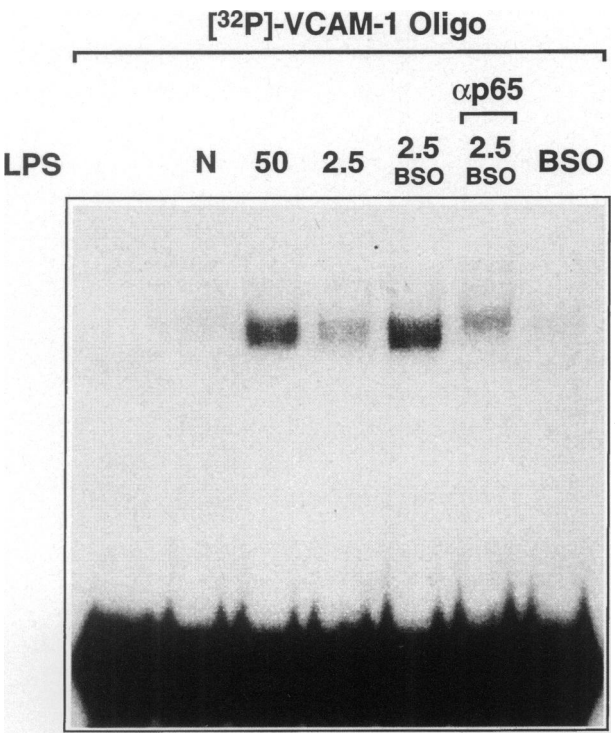

Figure 4. BSO potentiates LPS-induced NF-κB activation and interaction with proximal VCAM-1 promoter. EMSA using extracts from the renal cortex of mice treated with 50 μg of LPS demonstrates the interaction of NF-κB with the ^{32}P-labeled VCAM-1 oligonucleotide. Pretreatment with BSO followed by 2.5 μg of LPS induces a complex formation of equal intensity to that of 50 μg of LPS. This nucleoprotein complex is eliminated using antibodies to the p65 subunit. Administration of BSO alone does not activate NF-κB. The **first lane** represents radiolabeled oligonucleotide run without nuclear extract, as evidenced by the absence of a specific nucleoprotein complex with electrophoretic mobility slower than that of the probe itself.

of hemorrhagic cystitis caused by cyclophosphamide and ifofamide.[14] A recent report indicates its successful use as a chemopreventative agent in phase I clinical trials.[21] Our demonstration of the ability of NAC to down-regulate VCAM-1 expression by inhibiting the activation of NF-κB suggests that this antioxidant may be useful in the treatment of glomerular mesangial disorders and perhaps other pathophysiological settings in which signal transduction is mediated through NF-κB.[12,22]

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References

1. Wuthrich RP: Vascular cell adhesion molecule-1 (VCAM-1) expression in murine lupus nephritis. Kidney Int 1992, 42:903–914
2. Fries JWU, Williams AJ, Atkins RC, Newman W, Lipscomb MF, Collins T: Expression of VCAM-1 and E-selectin in an *in vivo* model of endothelial activation. Am J Pathol 1993, 143:725–737
3. Khachigian LM, Collins T, Fries JWU: Nuclear factor-κB mediates induction of vascular cell adhesion molecules-1 in glomerular mesangial cells. Biochem Biophys Res Commun 1995, 206:462–467
4. Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T: Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine-inducible enhancers. FASEB J 1995, 9:899–909
5. Schreck R, Rieber P, Baeuerle PA: Reactive oxygen intermediates as apparently widely used messengers in the activation of NF-κB transcription factor and HIV-1. EMBO J 1991, 10:2247–2258
6. Marui N, Offermann MK, Swerlick R, Kunsch C, Rosen CA, Ahmad M, Alexander RW, Medford RM: Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. J Clin Invest 1993, 92:1866–1874
7. Aruoma OI, Halliwell B, Hoey BM, Butler J: The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydoxyl radicals, superoxide, and hypochloric acid. Free Radical Biol Med 1989, 6:593–597
8. Schreck R, Baeuerle P: A role for oxygen radicals as second messengers. Trends Cell Biol 1991, 1:3942–3948
9. Shan Z, Tan D, Satriano J, Silbiger S, Schlondorff D: Intracellular glutathione influences collagen generation by mesangial cells. Kidney Int 1994, 46:388–395
10. Drew R, Miners JO: The effect of buthionine sulfoximine (BSO) on glutathione depletion and xenobiotic biotransformation. Biochem Pharmacol 1984, 33:2989–2994
11. Molitor JA, Walker WH, Doerre S, Ballard DW, Greene WC: NF-κB: a family of inducible and differentially expressed enhancer-binding proteins in human T cells. Proc Natl Acad Sci USA 1990, 87:10028–10032
12. Müller JM, Löms Ziegler-Heitbrock HW, Baeuerle PA: Nuclear factor-κB, a mediator of lipopolysaccharide effects. Immunobiology 1993, 187:233–256
13. Shah SV: Role of reactive oxygen metabolites in experimental glomerular disease. Kidney Int 1989, 35:1093–1106
14. Morgan LR, Holdiness MR, Gillen LE: N-acetyl-cysteine: its bioavailability and interaction with ifosamide metabolites. Semin Oncol 1983, 10:56–61
15. Schulze-Osthoff K, Beyaert R, Vandevoorde V, Haegeman G, Fiers W: Depletion of the mitochondrial electron transport abrogates the cytotoxic and gene-inductive effect of TNF. EMBO J 1993, 12:3095–3104
16. Anderson MT, Staal FJT, Gitler C, Herzenberg LA: Separation of oxidant-initiated and redox-regulated steps in the NF-κB signal transduction pathway. Proc Natl Acad Sci USA 1994, 91:11527–11531
17. Tetsuka T, Srivastava SK, Morrison AR: Tyrosine kinase inhibitors, genistein and herbimycin A, do not block IL-1β-induced activation of NF-κB in rat mesangial cells. Biochem Biophys Res Commun 1996, 218:808–812
18. Schieven GL, Kirihara JM, Myers DE, Ledbetter JA, Uckun FM: Reactive oxygen intermediates activate NF-κB in a tyrosine kinase dependent mechanism and in combination with vanadate activate the p561ck and p59fyn tyrosine kinases in human lymphocytes. Blood 1993, 82:1212–1220
19. Multicenter Study Group: Long term oral acetylcysteine in chronic bronchitis, a double-blind controlled study. Eur J Respir Dis 1980, 61:93–108
20. Prescott LF, Ballantyne A, Park J, Adriaensens P, Proudfoot AT: Treatment of paracetamol (acetaminophen) poisoning with N-acetylcysteine. Lancet 1977, 2:432–434
21. Pendyala L, Creaven PJ: Pharmacokinetic and pharmacodynamic studies of N-acetylcysteine, a potential chemopreventative agent during a phase I trial. Cancer Epidemiol Biomarkers Preven 1995, 4:245–251
22. Schreck R, Meier B, Mannel DN, Dröge W, Baeuerle PA: Dithiocarbamates as potent inhibitors of NF-κB activation in intact cells. J Exp Med 1992, 175:1181–1194